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A STANDARD METHOD FOR MOUNTING WHOLE ADULT LEPIDOPTERA ON SLIDES UTILIZING POLYSTYRENE PLASTIC

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COMPARATIVE MORPHOLOGICAL STUDIES of insects are greatly facilitated if the material is prepared and mounted in a way that permits its easy manipulation and rapid, yet detailed, examination. Mounting specimens dry, on pins, has been the method of preference of Lepidopterists virtually exclusively, but it does not permit detailed examination of integumentary features that are of great taxonomic importance. Attempts to interpret the relationships of higher categories in the lepidoptera entirely on the basis of structure externally visible on such specimens have, in many cases, led to errors and have confused the taxonomy of many groups.

Although most workers are willing to utilize slide mounts for limited parts of the body, especially the genitalia, they have not extended the treatment to the whole animal and prefer to keep their material in fluids, usually alcohol. While alcoholic specimens allow the greatest amount of freedom of examination, considerable time and patience is spent simply handling them—unstoppering vials, pouring alcohol, restoppering vials, etc. As a result, a worker may hesitate to make the innumerable comparisons and recomparisons requisite to phylogenetic analysis. Furthermore, since the specimen must be examined with relatively low power dissecting microscopes, many fine details, such as microchaetae and other minute sense organs, surface textures, striae, spicules, spiracular structure, etc., are not visible, or at least not clearly observable. Among the other disadvantages of alcoholic specimens are that they do not keep stains well, are subject to evaporation and destruction from drying and suffer damage due to repeated handling.

Slide mounts, on the other hand, permit very detailed observation and are very easily handled. Admittedly, they allow the specimen to be viewed only from a limited number of aspects, but if prepared in a careful manner, such as that described below, most of the integumentary anatomy is available for inspection. Slides may be stacked as high as 4 or 5 deep and the specimens readily compared by focusing up and down with a dissecting microscope or detailed drawings rapidly prepared for the same purpose with a microprojector. In addition, they may be permanently stained, are subject to destruction only by breaking,

and may be handled indefinitely without wear. Even fairly large Lepidoptera (e.g., smaller *Catocala, Vanessa*) may be placed on slides but I do not recommend it for very large species (e.g., saturniids, sphingids).

The details of the method outlined below were worked out on noctuid moths of medium size with relatively simple genitalia. Yet I believe that most groups of Lepidoptera may be similarly treated, although, of course, certain groups may require special handling because of extremely small size or peculiar modifications, especially in the genitalia.

The general treatment below might be applied advantageously to many insect orders (especially Neuroptera, Trichoptera, Diptera, etc.). All stages of mosquitoes are beautifully accommodated and I have had promising results with Collembola, a group notoriously difficult to mount on slides. The latter usually shrivel badly in balsam and eventually deteriorate if placed in aqueous media, apart from being flattened or otherwise distorted.

THE USE OF POLYSTYRENE PLASTIC

Whole mounts of most insects require fairly thick preparations. Workers who have tried to make such preparations with the usual natural (Canada balsam, Euparal), synthetic (Permount, Diaphane), or mixed aqueous (PVA, Hoyer's) media find that there are certain difficulties: These media, when applied in large amounts take a very long time to harden, specimens invariably move in them from their desired positions during the hardening period (even if the laborious and time consuming "layering" or "build-up" technique is used), and they change color or deteriorate after a time.

Polystyrene plastic[1] (hereafter referred to simply as "plastic"), however, primarily because of its setting qualities, is the ideal medium for holding dissected parts in their desired positions on slides. Because it gels evenly and rapidly throughout, any specimen, regardless of shape or center of gravity, may be orientated perfectly in any position. Further advantages of plastic over the media mentioned above are its greater transparency and resistance to discoloring, and its thorough hardness and permanency when finally cured. In addition, it has the desired qualities of being its own clearing agent and of slightly intensifying certain stains. It has one disadvantage at present; it cannot be dissolved gradually and without fragmenting after complete hardening by any agent I know and therefore a specimen cannot be removed once mounted. This is a serious drawback, however, only with scarce material which might need to be remounted for study from a different aspect. The methods described in this paper are intended primarily for morphological and phylogenetic studies where abundant material is available. In any case, since slides are rarely remade unless very poorly prepared

[1]Known commercially by many trade names, this is the type used for embedding biological specimens and other items by hobbyists.

in the first place, the insolubility of plastic should not limit its use. Preparations on broken slides may be soaked off in cellosolve and glued onto new slides with a thin layer of plastic.

The steps involved in using plastic as a medium for thick microscope slide mounts are as follows:

1. The specimen, after dissection and dehydration in up to 95% ethanol (see directions below), is transferred to a mixture of 50% acetone and 50% uncatalyzed plastic (because of the difference in density between these components they do not mix readily—mixing may be accomplished by shaking them together in a vial for a few seconds). Since the acetone evaporates rapidly from the mixture, its container must be kept covered as much as possible. The mixture clears and completely dehydrates the specimen; the specimen should remain in it about fifteen minutes.

2. While the specimen is clearing, a slide is prepared to receive it in the following manner: Uncatalyzed plastic is dropped onto the slide and spread out over an area the size of the cover slip. Spreading is done with a glass rod (3mm. diameter), which has been previously dipped into catalyst. The rod is used to apply and thoroughly mix the catalyst into the plastic. The depth to which the rod is dipped determines the amount of catalyst picked up. I find that dipping it about 5mm. provides the right amount of catalyst to gel the thickest layer of plastic that can be spread over a 22mm. square area (size of standard, square cover slip) without running over. Enough plastic should be applied to just cover the thickest part of the specimen. If the specimen is very thick and the viscosity of the plastic is not enough for it to stand level with the specimen, more layers must be added.

3. The specimen is next transferred to the plastic on the slide and generally oriented.

4. The slide is then placed on a warming plate, previously heated to about 130°F. to accelerate the gelling of the plastic. While the preparation is on the plate, it is viewed with the low power of a dissecting microscope and the specimen appropriately oriented. The specimen is held in its desired position with dissecting needles until the plastic gels enough to firmly anchor it. At this temperature, gelling will begin rapidly and will be far enough advanced to hold the specimen in about 7-10 minutes[2]. Impending gelation can be noted by the cessation of movement in the plastic revealed by dust particles present in it.

5. The slide is allowed to remain on the warming plate about 15 minutes to ensure thorough gelation.

6. The preparation is then ready to receive a cover slip if the first layer of plastic was sufficient to cover the specimen. If not, more layers must be added in the same manner as the first.

7. Glass chips or other cover slip supports are unnecessary and should not be used since they hinder the settling of the cover slip on the slightly shrinking plastic, and may cause a broken preparation.

8. The cover slip is prepared by covering it with just enough catalyzed plastic (applied with glass rod as above) to spread over its entire area when inverted and placed on the specimen. When the cover slip is in place the whole preparation is returned to the warming plate for gelling. The heat also helps the plastic under the cover slip to spread. If too little plastic was applied, additional amounts may be added at the side of the cover slip until all the spaces are filled. Any excess plastic may be wiped off from around the edges or, if gelling has occurred, trimmed with a blade.

9. Complete hardening and curing of the whole preparation can be accomplished by leaving it overnight in an oven at about 100°F. or can be allowed to take place slowly at room temeprature.

[2]This figure varies according to the formulation and age of the plastic. I find these times average but they may be longer especially for formulations designed for long shelf life.

STANDARD DISSECTION AND MOUNTING

Four slides are required to hold the various parts of the whole insect (fig. 1): 1. The main part of the body including the two halves of the head, thorax and abdomen are put on the first slide (the tegulae, antennae and basal portions of the right wings being dissected away—see below) (fig. 1:1); 2. A second slide holds the legs, basal portions of the right wings, antennae and tegulae (fig. 1:2); 3. The wings occupy a third slide (fig. 1:3); 4. The genitalia are put on a final slide (be they male (fig. 4b) or female (fig. 4a).

These main parts are dissected and mounted as follows:

Preliminary general dissection

The following instructions apply to specimens mounted on pins. Specimens in alcohol or otherwise preserved are excused from the impertinent steps.

1. The left pair of wings are broken off at their bases and the right pair cut off transversely with scissors beyond the end of the frenulum. The wings, with the data labels removed from the pin and a slide reference label, are set aside in a cellophane envelope.

2. The body of the insect, still on its pin, is wetted in 75% ethanol and then boiled in water for 5 minutes. This softens it and allows the pin to be removed easily.

3. The body is next transferred to a solution of 10% potassium hydroxide which is heated to near boiling in a small beaker or test tube suspended in a water bath. The specimen is left in the solution until thoroughly macerated (15 minutes).

4. After maceration, the body is transferred for dissection and cleaning to distilled water in a watch glass, preferably one with a slightly convex bottom.

5. Next the genitalia are removed. In the male this is done by severing the membrane between abdominal segments VII and VIII (or further cephalad to include special modifications on more proximal abdominal segments if present). The female is treated likewise, but by severing the membrane between segments VI and VII. The genitalia are rubbed clean of scales (I like to use a pointed applicator stick for this; others may prefer a fine camel's hair brush or like instrument) and transferred to clean distilled water.

6. The legs are now removed (coxae and trochanters should remain intact), rubbed clean of scales and transferred to clean water.

7. The body itself (head, thorax with bases of legs, and abdomen minus genitalia) is finally bisected with a sharp, single-edged razor blade. Great care must be taken at this step to insure as perfect a bisection as possible. The specimen is held ventral side up in the watch glass and a mid-ventral, longitudinal slit is made at the posterior end of the abdomen with scissors. The razor blade is then brought down on the mid-ventral line guided by this slit, the coxae and the two halves of the proboscis between which its edge is placed. The blade is first used to compress the body gently and then pressed firmly against the bottom of the watch glass and rocked to and fro to make the final cut. The slightly convex bottom of the watch glass helps to make a clean cut. After the halves of the body are separated, cleaned and rubbed free of scales, they join the other parts in distilled water.

8. Dehydration in 75% and 95% ethanol follows (if no aqueous stain is used—see "STAINING"). The female genitalia and phallus of the male genitalia go directly to 95% ethanol to harden their membranous parts. This is done in a special manner as described below.

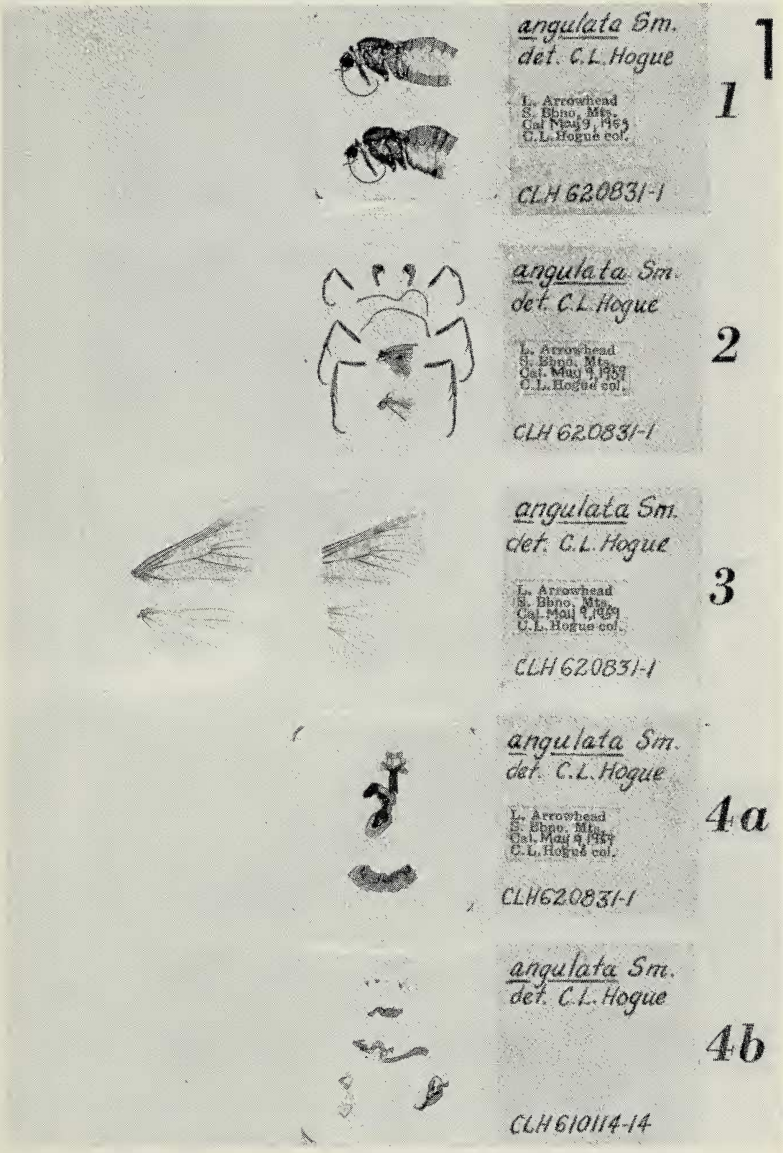
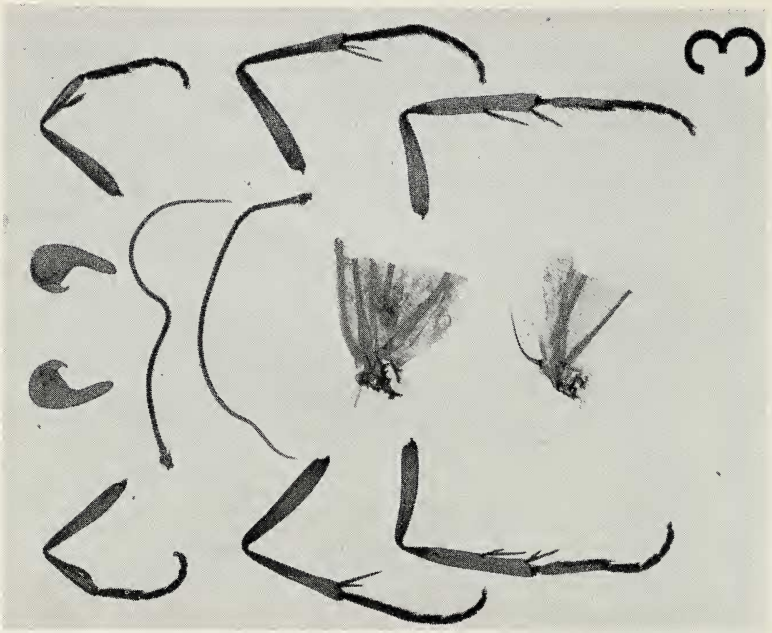


Fig. 1: Set of slides (*1* - *4*) holding a whole specimen (*4a* example a female; *4b* a male).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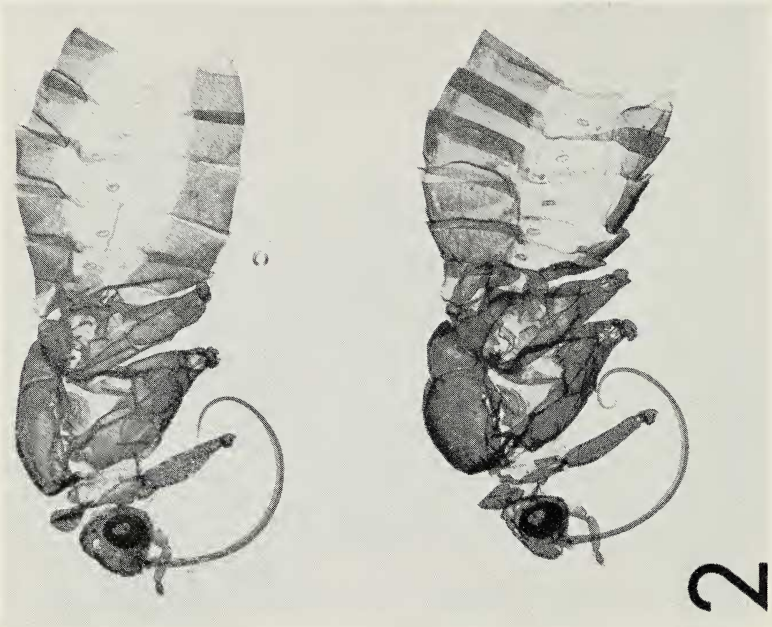
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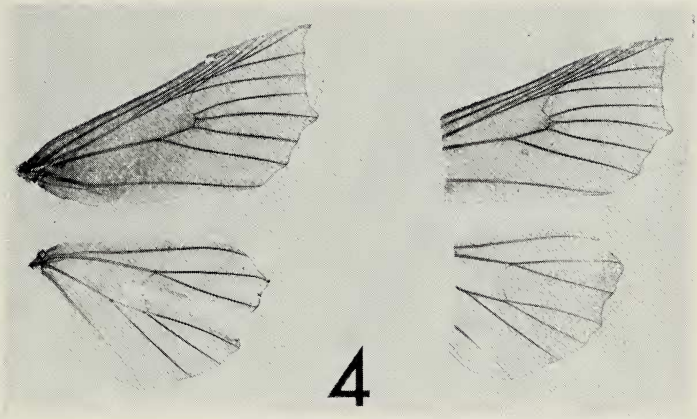


Figs. 2-6: Close-up views of slides shown in fig. 1 to show detail of arrangement of parts. Fig. 2: Main body, bisected. Fig. 3: Miscellaneous parts. Fig. 4: Wings. Fig. 5: Female genitalia. Fig. 6: Male genitalia.

9. The wings, previously set aside in a cellophane envelope, are bleached for about 2 or 3 minutes (at least long enough for the veins to become well defined) in 5% sodium hypochlorite (full strength household bleach—Purex, Chlorox), washed in distilled water, and dehydrated by passage through 75% and 95% ethanol.

If it is desired that the veins be stained a totally different procedure is necessary:

After wetting in ethanol and soaking for 20-30 minutes in hydrogen peroxide (3%), the scales are carefully rubbed off with a blunt camel's hair brush. The denuded wings are then washed in distilled water and transferred to the stain. A long destaining period must follow in order to decolorize the membranes.

The dissected parts, now in 95% ethanol, are ready for fine, detailed dissection and cleaning (and staining with non-aqueous stains if desired—see "STAINING") before mounting in plastic on slides. I find that these two jobs are best done in the 95% ethanol; since the specimen has become fairly brittle, sclerites are easily separated and remaining unwanted scales easily flaked off.

Final dissection and mounting

The different parts are treated in various ways. Reference to the figures will clarify the descriptions (including terminology) of the fine points of dissection and orientation on the slide.

Slide 1: Main body (figs. 1:1;2)

The two halves of the main body of the specimen require only minor alteration before being transferred to the clearing mixture. The tegulae and antennae are removed and the bases of the right pair of wings are carefully dissected away with the axillary sclerites. These parts are mounted with the legs (see "Slide 2"). If the specimen is a noctuoid moth, the ventral portion of the second phragma is also cut away with fine scissors to allow an unobstructed inner view of the tympanal area. Similar special treatment may be necessary with other taxa.

The halves are mounted on the slide, one with its outer surface up and the other (the one with the cut phragma if noctuoid) with its inner surface up.

Slide 2: Legs, etc. (figs. 1:2;3)

The legs, bases of wings (with axillary sclerites), tegulae and antennae are all mounted together on the same slide since they are of about the same thickness and do not orient well if left attached to the main body. Usually these parts require no further dissection although sometimes one may desire to separate the components of the pretarsus or the antennae. Their orientation is best explained by the figures.

Slide 3: Wings (figs. 1:3;4)

No special treatment is needed for the wings. After dehydration they are simply mounted flat in the plastic (which also clears them). If too large to fit on a standard microscope slide, they may be accommodated by a large sized slide (2″ x 3″). If very large, they may have to be cut up into sections.

Slide 4: Genitalia — female (figs. 1:4a;5;7)

The following procedure is considerably modified from that usually employed by lepidopterists. The conventional procedure is to leave abdominal segment VII attached and to mount the entire undissected complex ventral side up or laterally on the slide. This, however, has the following undesirable features: (1) segment VII, still attached, obscures detailed viewing of the structures beneath, (2) if mounted with the ventral side up the venter of segment VIII is plainly visible but the pleura, which also may possess important characters, are not. Furthermore, only the ventral edges of the ovipositor lobes are in plain view; their profile, patterns of sclerotization and chaetotaxy are impossible to see. If mounted laterally, the reverse viewing difficulties are present. (3) The whole complex is cylindrical in general shape and rolls easily, making it hard to position in a standard way.

By mounting the female genitalia as described below, these difficulties are surmounted. The steps involved are as follows (fig. 7 shows them partly completed):

1. The genitalia have been freed from the remainder of the abdomen and cleaned of most of their scales (see above, "Preliminary general dissection").

2. Segment VII is removed by splitting it laterally and tearing it off. The tear should follow the line of the membrane between it and the following segment. Care must be taken not to damage the sclerites around the ostium bursae.

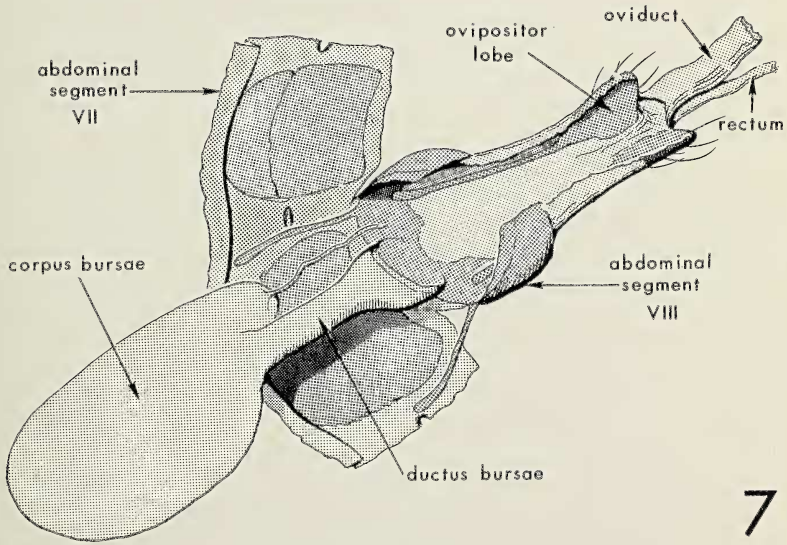


Fig. 7: Oblique dorsal view of female genitalia showing principal structures in state of partial dissection (diagrammatic).

3. Next, the genitalia proper are slit mid-dorsally, a cut being made first with scissors through segment VIII and continued by tearing caudad to the tip between the ovipositor lobes.

4. The genitalia are then opened up, the oviduct and rectum are pulled up and cut free, and the whole complex cleaned.

5. The bursa copulatrix is next inflated with water in much the same manner as the vesica of the male phallus (see below). The cannula is inserted into the ostium bursae and passed down the ductus bursae. The stream of water from the syringe cleans the corpus bursae internally and expands it into its full shape. Sometimes a tiny hole is needed to allow the escape of the fluid and debris. It is usually not necessary to go through a hardening process with the bursa copulatrix as is the case with the male phallus vesica since it generally holds its shape better than the latter.

6. Staining with aqueous stains follows if desired.

7. Preparatory to dehydration in 95% ethanol the genitalia are opened again and placed flat (inner surface down) in a stender. A small piece of glass (broken microscope slide) is placed over the opened portion (not over corpus bursae!) to hold it flat while the water is withdrawn from the vessel and replaced with 95% ethanol. The same may be done for segment VII, previously removed. It may be necessary, if not already done, to punch a small hole in the corpus bursae before adding the ethanol if the ductus bursae is so narrow that it would prevent the free exchange of liquids. Otherwise, the diffusion gradient—ethanol diffusing out faster than water diffusing in—may cause the collapse of the bursa.

8. Final cleaning may be carried out after dehydration. The preparation is then ready for clearing and mounting.

Slide 4: Genitalia – male (figs. 1:4b; 6; 8-10)

Lepidopterists disagree on dissection procedures and orientation of the male genitalia on slides. The majority prefer to leave all the elements intact and mount the complex ventrally with the valves spread open as widely as possible, the least troublesome method. Others also do not separate the parts but mount the whole complex laterally. Still others dissect various parts away, often only the phallus whose vesica may or may not be inflated and the main orientation is either ventral or lateral.

These variations may be dictated by either the whims of the investigator or the nature of the material, i.e., the positions of the characters of greatest significance. Too often it is the former. Obviously, the only scientific and rational approach to the problem is to dissect and mount many specimens in many ways, even keeping some unmounted in fluids. Yet when time is limited, and many comparisons are to be made, a single, standard method must be chosen. For this, I prefer to mount the main structure of the genitalia laterally, with one valve and other elements such as the juxta, anellus, etc. removed and mounted separately. Furthermore, I always inflate the vesica of the phallus; though this is a time consuming task, the results are well worth it.

Even apart from the conditions just mentioned, I believe that this procedure is superior to the undissected ventral mount for morphological study since there are generally better, undistorted viewing aspects for the majority of characters and additional characters are revealed. Specifically, (1) the uncus is natural in shape and position instead of

being folded or angled if long, or viewed on end if short. Its entire profile is observable in plane view; (2) the tegumen is untwisted (otherwise usually resulting from the disturbed uncus); (3) the overall shape, lobes and spines etc. of the vesica are fully exposed, not compressed into the body of the phallus; (4) the anellus is not obscured by the juxta (and phallus too if left intact); (5) the valves (at least the one dissected away and usually also the one intact) lie completely flat so that their median structures are not distorted by foreshortening as is usually the case in a ventral mount.

The steps followed in dissecting the male genitalia are as follows:

1. The genitalia have been freed from the remainder of the abdomen and cleaned of most of their scales (see above, "Preliminary general dissection").

2. The phallus is removed by grasping it at the tip, i.e., posterior end protruding between juxta and anellus (fig. 8), and pulling gently to the rear (fig. 9). If the phallus does not so protrude, the forceps may need to be inserted a short distance into the canal inclosing it to gain a hold. If the phallus is withdrawn properly and carefully, the manica will turn wrong side out and the ductus ejaculatorius will pull out through the hole in it (fig. 9).

If staining with aqueous stains is desired at this point, the main portion of the genitalia should be set aside in distilled water. Otherwise, they may go directly into 95% ethanol.

3. The ductus ejaculatorius should now be snipped off with a pair of fine scissors a short distance from the aedeagus.

4. Next comes the preliminary inflation of the vesica. This is accomplished by first teasing as much as possible of the vesica out of the aedeagus with fine forceps and then inflating it with a hypodermic syringe filled with distilled water. I find that a 2cc. syringe tipped with a glass cannula (in preference to a regular hypodermic needle which does not permit the variety of sizes needed) best for this work. The cannula is inserted into the ductus ejaculatorius (fig. 10) and pressure applied until the vesica completely inflates and everts. It is usually necessary to crimp the ductus with forceps against the cannula at the point of insertion to prevent leakage and the phallus from coming free under the hydraulic pressure.

5. The vesica is next hardened to preserve its fully inflated shape (fig. 10). This step follows staining if aqueous stains are used. Several steps are necessary in this process:

a. The phallus is placed in clean distilled water in a watch glass.

b. A short length (1 cm.) of silk surgical suture (cardiovascular 5-0 or 6-0) is tied into a loose, simple knot and also put into the watch glass.

c. The hypodermic syringe is filled with 95% ethanol.

d. The cannula is inserted as above. This may be difficult since the alcohol rapidly diffuses out of the tip setting up a strong current which constantly carries the phallus away.

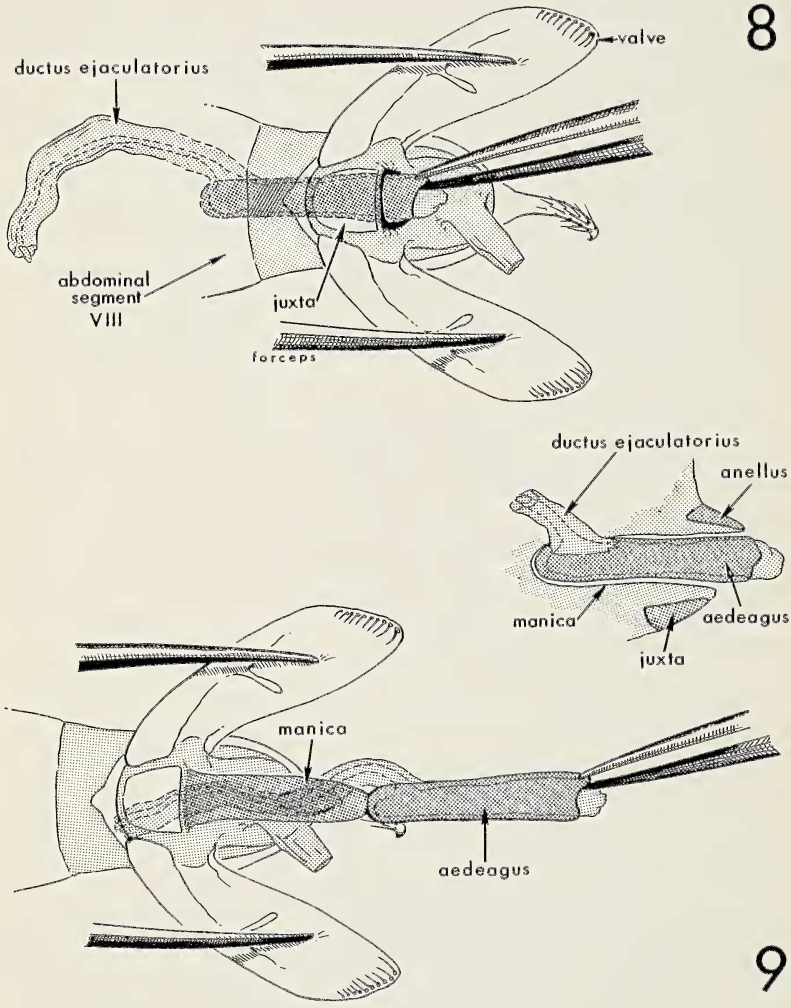
e. After the cannula is properly inserted the phallus is tied to it with the suturing thread (around the center of the aedeagus) by slipping the loose knot over it and drawing it tight with forceps (fig. 10). Some kind of clamp or modelling clay is useful at this point to hold the syringe while both hands are being used to tie the knot.

f. The water in the watch glass is now withdrawn and quickly replaced with 95% ethanol.

g. Pressure is exerted with the syringe and maintained for a short while (about 3 minutes usually suffices) until the vesica is thoroughly dehydrated and hardened. Leakage of the ethanol from the end of the vesica is prevented by clamping it with fine forceps (fig. 10).

h. The phallus is slipped off the cannula and the suturing thread untied and removed.

i. The phallus joins the rest of the genitalia in 95% ethanol.



Figs. 8-9: Ventral views of male genitalia showing principal parts and procedure for removal of phallus. Insert showing relationship of components of phallus complex in lateral, sectional view. (All figures diagrammatic).

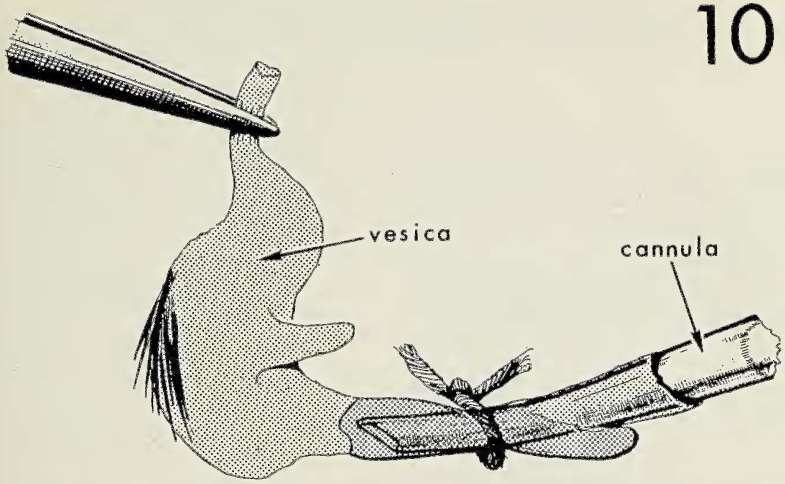


Fig. 10: Phallus of male genitalia properly prepared for inflation and hardening of vesica (diagrammatic).

6. Further dissection, if desired (at least segment VII should be removed) and final cleaning may now be carried out in preparation of clearing and mounting.

STAINING

Only a few general remarks regarding stains will be made here. Each worker has personal preferences and many stains are in popular use, among them acid fuchsin, mercurochrome, and safranin. I have always found the first the most satisfactory. Acid fuchsin is taken up in the greatest amounts by the sclerotized parts of the integument leaving membranous parts clear. Thus sclerites are clearly defined and easily observed and drawn. Tiny sclerotic processes in membranes also show up very distinctly. I have not found the other stains mentioned to act in this manner.

Acid fuchsin is an aqueous stain, i.e., it is soluble in water and must be applied to a fully hydrated specimen. I usually apply it after preliminary dissection using a 5% solution diluted 1 drop to 10 ml. Overnight is usually long enough to leave the parts in the stain. If not enough stain is taken up in this time more is added until the specimen is deeply tinted. Staining is always followed by destaining in distilled water to clear the membranes and obtain the desired differentiation.

Non-aqueous stains may be applied to the specimens after they have been dehydrated in 95% or absolute ethanol.